SUPPLEMENTAL MATERIAL

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Restoration of Cardiomyogenesis in Aged Mouse Hearts by Voluntary Exercise

Supplemental Methods

Animal studies

20 months old aged C57BL/6J male mice were used for the primary investigations. For additional experiments and comparison to young mice, male mice at the age of 10-12 weeks were also investigated. This first study about exercise-induced cardiomyogenesis in aged mice had to be limited to one gender for reduction of animals given the overall difficulty of MIMS and it's time- and expense factor in addition to potential gender-differences that were not the focus of this current study. Additionally, as described in detail below, we had extensive experience with using Y-chromosome fluorescent in situ hybridization for ploidy analyses. All mice were studied and cared for in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Faculty of Arts and Science, Harvard University (protocol number 16-05-273), Massachusetts General Hospital (protocol number 2015N000029), and regional authorities (Regierungspräsidium) Karlsruhe, Germany. No a priori inclusion/exclusion was applied, and a simple randomization was used for all animal studies. Results were analyzed in a blinded fashion.

Running exercise protocol and ^{15}N-thymidine labeling

Mice were randomly assigned to exercise or sedentary control groups. Aged mice with visible tumors were excluded from the study; otherwise no a priori exclusion criterion was applied. Exercise and ^{15}N-thymidine labeling were conducted as previously described[10]. In brief, an osmotic minipump (Alzet) containing ^{15}N-thymidine (Cambridge Isotopes) was implanted subcutaneously at 20 months of age, delivering ^{15}N-thymidine at a rate of 20µg/h. The pump was exchanged weekly for 8 consecutive weeks. During that time, mice were housed individually in plexiglass cages (36 L x 20 W x 15 H cm) with the exercise group having free voluntary access to a running wheel (diameter 11.4 cm; Mini-Mitter, Starr Life Science, USA) equipped with a tachometer to monitor daily activity. Sedentary control mice were housed under the same conditions without a running wheel and all mice were sacrificed after 8 weeks.

Echocardiography

Mice were sedated with 0.1–0.5% inhaled isoflurane for echocardiography, with the dose titrated to maintain heart rates of >450 beats/min for acquired images. Mice were placed on a heating pad, and echocardiograms were obtained at mid-papillary level using a Vevo770 imaging system (Visualsonics, Toronto, Ontario, Canada). M-mode was used to measure left ventricular interventricular septal wall thickness, left ventricular posterior wall thickness, and left ventricular internal diameter during both systole and diastole. Fractional shortening (%) was calculated with the Visualsonics software package.

Tissue harvesting
Body weights were measured for every mouse before deep sedation with Ketamin/Xylazin (80mg/12mg/kg). Hearts were then excised, and heart weights were recorded, as well as tibia lengths and lung weights. The right ventricle was removed, and the left ventricle was subdivided along the short axis. The section at the level of the papillary muscle was reserved for histology and imaging analysis (see below), while the apical and basal section were flash frozen and used for RNA analysis. All mice were sacrificed around the same time of day.

RNA isolation, RNA Sequencing and quantitative real-time PCR (qPCR)
RNA from tissues and cell samples was isolated using Trizol (Invitrogen #15596018) and phase separation, followed by column purification according to the manufacturer's instructions (Zymo Research #R2052). Reverse transcription was carried out using the Universal cDNA Synthesis kit (Exiqon). For RNA Sequencing, libraries were prepared using polyA-selected RNA and sequenced on an Illumina HiSeq 2500 instrument in High-Output mode as paired end 100. The raw sequence image files in form of .bcl were converted to fastq format using bcltofastq v1.8.4 and processed with bioinformatics workflow as follows: Illumina RNA-seq reads were pre-processed with Flexbar 3 [PMID: 28541403] for quality clipping and adapter removal. We used Bowtie2 [PMID: 22388286] and mouse reference transcripts (rRNA, tRNA) to subtract t/rRNA reads *in silico*. All the remaining reads were aligned against the mouse genome using STAR (2.6.0c) [PMID: 23104886], guided by the EnsEMBL v96 reference annotation (mm39/GRCm39). We performed gene and transcript abundance estimation with Stringtie 1.3.5 [PMID: 25690850] and generated a read count table (using the prepDE.py script). Differential gene expression analysis was performed with edgeR (v3.24.3, [PMID: 22287627]). Briefly, we performed significance testing using a two-factor design matrix with age and exercise plus interaction term as covariates. All gene loci with an FDR <= 0.05 in any tested model term were retained as significant. The functional enrichment analysis was performed using g:Profiler (version e104_eg51_p15_3922dba) with g:SCS multiple testing correction method applying significance threshold of 0.05, including genes of interest from the exercise coefficient (FDR <0.05).[16] We enhanced our interpretation of the RNA-seq data through gene set enrichment analysis (GSEA) for the DGE model interaction term (Old:Exercised). GSEA evaluates differences in gene rankings as defined by ordering gene expression fold changes over pathway annotations. We employed the ReactomePA R package and included all genes with an unadjusted p-value of 0.05 in this analysis.[43] Among the top ranking pathways, we noticed cell cycle related records (e.g. rank 33, Mitotic prophase), which show an enrichment for up-regulated genes in aged and exercised animals.

For qPCR analysis, 1µg of isolated RNA was used for cDNA synthesis with reverse transcription reactions as described by the manufacturer (Applied Bioscience #4368814) before qPCR was conducted using SYBR-green (Biorad #1725124) on either a ViiA7 (ABI) or Bio-Rad CFX384 Real-Time System. Thermocycle conditions were set to 40 cycles, 3s at 96°C (denaturation) and 30s at 60°C (annealing/extension) each cycle. qPCR analysis from adult cardiomyocyte and non-cardiomyocyte RNA preparations was performed using TaqMan probes for cardiac troponin T2 (*Tnnt2*, Mm01290256_m1) and

collagen type 1 alpha 1 (*Col1a1*, Mm0080166_g1), with TATA-binding protein (*Tbp*, Mm01277042_m1) used as the housekeeping gene on a Bio-Rad CFX384 Real-Time System.

The following primers were used:

m CEBPβ f	GGGGTTGTTGATGTTTTTGGT
m CEBPβ r	TCGAAACGGAAAAGGTTCTCA
m CITED4 f	CCTGGCATACGGCTCCTTC
m CITED4 r	AGACTGCAGGTGCGTGCTAC
m Hipk1 f	CAGCATCAGCCAATCATC
m Hipk1 r	TTGTCCTCTTCTTCATCAGT
m Hipk2 f	TACGAGCAGACCATCATC
m Hipk2 r	GTTCTCAATCTCCTCACTCT
m+r αMHC f	ACATCAGTCAGCAGAACA
m+r αMHC r	TTCCTCTAGCCTCTCACT
m+r βMHC f	GCTGTTATTGCTGCCATT
m+r βMHC r	TTATCATTCCGAACTGTC
m+r Nppa f	GAGCAAATCCCGTATACAGTGC
m+r Nppa r	ATCTTCTACCGGCATCTTCTCC
m+r Nppb f	GCTGCTGGAGCTGATAAGAGAA
m+r Nppb r	GTTCTTTTGTAGGGCCTTGGTC
m PGC1α f	CCCTGCCATTGTTAAGACC
m PGC1α r	TGCTGCTGTTCCTGTTTTC
m+r RCAN1.1 f	GACCCGCGCGTGTTC
m+r RCAN1.1 r	TGTCATATGTTCTGAAGAGGGATTC
m+r RCAN1.4 f	CCCGTGAAAAAGCAGAATGC
m+r RCAN1.4 r	TCCTTGTCATATGTTCTGAAGAGGG
m+r TBP f	CCCTATCACTCCTGCCACACCAGC
m+r TBP r	GTGCAATGGTCTTTAGGTCAAGTTTACAGCC
m CCND1 f	GCGTACCCTGACACCAATCTC
m CCND1 r	CTCCTCTTCGCACTTCTGCTC
m CDCA2	AAGGAATCCAACCTCTGTGATGA
m CDCA2	ACAGCCTGGTGTTTTACAGTTTT

Online Table I: Primers (mouse = m; rat = r)

Multi-isotope imaging mass spectrometry (MIMS) and nucleation analysis

Midventricular heart sections were fixed with 4% paraformaldehyde (PFA) and embedded in London resin (LR) white. A middle section (0.5μm) was mounted on a silicon chip for MIMS analysis and serial sections below and above, covering up to 100μm of tissue, were prepared for PAS staining (section thickness 1μm) or Fluorescent in situ hybridization (thickness 0.5 μm immediate five sections above and below chip). MIMS analysis was

conducted as previously described using a NanoSIMS 50L (Cameca).[10] ^{15}N-thymidine positive and negative cells were assessed, counted and identified as a cardiomyocyte (CM) or non-CM. To assess nucleation of each ^{15}N-thymidine positive CM, PAS- staining was used as previously described. [10] Briefly, slides were immersed in xylene for 2 x 30 min, rehydrated through graded alcohols, incubated in Periodic acid solution for 24h and then in Schiff's reagent for 48h. After washing, dehydration, clearing and mounting, slides were imaged on a NanoZoomer Whole Slide Scanner (Hamamatsu Photonics). Each ^{15}N-thymidine positive CM was identified in the adjacent PAS-images, cell membranes were outlined, and followed through all sections as long as the cell was present. The total number of nuclei identified in each cell was recorded. Based on PAS- images, adjustments were made regarding the assignment of cells to the CM or non-CM group if necessary. All analyses were performed by observers blinded to group assignment.

Fluorescent in situ hybridization (FISH)

FISH sections were incubated for 10min at 80°C in 1 M sodium thiocyanate, washed in PBS, treated for 2 min in 0.2% Triton X (in PBS) and then digested for 30 min at 56°C with 50µg/ml Proteinase K. Next the sections were rinsed with PBS, for 5 min post-fixed in 4% PFA/PBS and treated for 10 min with 50% formamide/ 4x standard saline citrate buffer at 37°C before being air-dried. Biotinylated-labeled chromosome Y paint (MCENY-10-BIO, Empired Genomics) was suspended in hybridization mix and applied to the air-dried sections, which were then sealed under glass with rubber cement, denatured for 3 min at 69°C and finally incubated at 42°C overnight. After, they were first washed three times with 2x standard saline citrate buffer and then two times for 10 min each with PBS/0.1% Tween (PBST) at room temperature. The sections were blocked with 10% goat serum in PBST and then incubated for 1 h in streptavidin- conjugated Alexa Fluor 488 (S32354, Invitrogen) before being washed with PBST again and mounted. They were imaged on a LSM510 Inverted Confocal (Zeiss LSM 510) and analyzed with Zen lite software (Zeiss).

Capillary density

To identify changes in vascularization upon exercise, we quantified the expression of the endothelial cell marker CD31 (PECAM1) and determined the capillary density (capillaries/cardiomyocyte). Mice who underwent the identical exercise/sedentary protocol as outlined above but were not used for MIMS and nucleation analysis were used. Hearts were flash frozen in OCT (optimal cutting temperature) as previously described.[44] 6µm sections were cut from the frozen tissue, air-dried on a coverslip for 5 min and then fixated for 10min in fresh 4% PFA. Immunofluorescent staining was conducted with an antibody against CD31 (BD Pharmingen #557355,) and wheat germ agglutinin (488- Oregon green, Thermo Fisher Scientific). Alexa Fluor (555-texas red) fluorescent dye (Thermo Fisher Scientific) was used as a secondary antibody. Images were taken on a Leica SP8 confocal microscope.

Masson's trichrome staining

Left ventricular fibrosis in aged sedentary and exercised mice was assessed using Masson's trichrome staining as previously described [45]. Briefly, paraffin sections (5 µm) were fixed at room temperature overnight in Bouin's fixative. After rinsing in tap water to

remove residual Bouin's fixative, sections were stained with a 1:1 mixture of hematoxylin A and B (A, catalog 88028, and B, catalog 88029; Thermo Scientific) for 10 minutes. Scarlet acid stain (catalog 26367-04, Electron Microscopy Sciences) was added for 10 minutes, followed by 5 minutes incubation in phosphomolybdic acid solution (catalog 26367-05, Electron Microscopy Sciences). The sections were then incubated in aniline blue solution (catalog 26367-06, Electron Microscopy Sciences) for 6 minutes and transferred to 1% acetic acid for 5 minutes. This was followed by dehydration in ethanol and xylene; the sections were then mounted in permount (catalog 17986-01, Electron Microscopy Sciences) and cured for 24 hours before imaging. Stained sections were imaged using Zeiss AxioScan imaging system at the Harvard Center for Biological Imaging. Total fibrosis was calculated using ImageJ (NIH) as previously described[45].

Neonatal rat ventricular cardiomyocyte isolation and culture, siRNA transfection and adenoviral infection

Neonatal rat ventricular myocytes (NRVMs) were isolated by enzymatic digestion of 1- to 2-day old neonatal rat hearts as previously described[46]. Cardiomyocytes were purified by pre-plating for 2 h at 37°C in 15cm dishes in F12-DMEM (gibco) containing 10% FBS. $0.5x10^6$/well NRVMs were plated in 6-well plates (Primaria) and cultured in F12-DMEM/10%FBS/5%Horseserum (HS) for 24h. Cells were synchronized by serum starvation for 18h before being treated with 100nM IGF-I (Peprotech) or 10µM Phenylephrine (Sigma Aldrich) for indicated timepoints.

All additional cell culture experiments were carried out with NRVMs purified by Percoll density gradient centrifugation. For protein and RNA analysis $0.5x10^6$/well purified NRVMs were plated in 6- well plates and cultured in F12-DMEM/10%FBS/5%HS for 18h. Cells were then transfected with 50nM RCAN1- or control- siRNA (Thermo Fisher #1330001 and #4390844) using Lipofectamin RNAiMax (Invitrogen), following the manufacturer's instruction except for a reduction of Lipofectamin to 4.5µl/well. 18h after transfection cells were treated with F12-DMEM/0% FBS or F12-DMEM/2%FBS for 24h. All media contained 1% penicillin/streptomycin (10,000U/10mg). Amplification of adenoviruses (Ad5 serotype) for expression of human RCAN1.1. and RCAN1.4. was carried out as described previously at an MOI of 50, LacZ adenovirus was used as a control at equivalent MOIs.[47-49]

Immunoblotting and immunofluorescence staining

Both tissue and cell samples were processed as previously described[14]. In brief, hearts were excised, washed in PBS, and left ventricular tissue was snap frozen and stored before homogenization in RIPA lysis buffer (#89901 Thermo Fisher Scientific) containing Proteinase/Phosphatase inhibitor (#78442 Thermo Fisher Scientific) using a tissue homogenizer. Samples were then centrifuged at 20,000rpm to precipitate debris and the supernatant was used immediately in further steps. NRVMs were harvested in RIPA buffer (+ Proteinase/Phosphatase inhibitor), scraped from the plate, and homogenized by repeated aspiration through a hypodermic needle. Protein concentration was measured using Pierce BCA assay (Thermo Fisher Scientific). Samples were combined with appropriately concentrated Laemmli buffer and boiled before SDS-PAGE (#5671095 Criterion, BioRad) followed by transfer to nitrocellulose membranes. Membranes were

probed with primary antibodies for Vinculin ((V9264, Sigma Aldrich) and RCAN1 (Anti-DSCR1, D6694, Sigma Aldrich). An appropriate secondary antibody conjugated to HRP (#7074S and 7076S, Cell Signaling) was used for detection and images were acquired using Vilber Fusion FX. Blots were analyzed using FusionCapt Advance (Vilber).

For immunofluorescent staining of NRVMs, coverslips were coated with Laminin/PBS (1:100, Sigma Aldrich L2020-1MG) in 24- well plates overnight before $8x10^4$ cells were plated per well. Transfection and treatment were conducted as described above. Cells were fixed in 4% PFA, and permeabilized in 0.5% Triton X-100. First antibody (α-sarcomeric actinin (Abcam #ab9465) and ki67 (cell signaling #9129)). Cardiac sections were also also fixed in 4% PFA, and permeabilized in 0.5% Triton X-100. First antibody staining (Vimentin Progen #GP53) as well as Wheat Germ Agglutinin staining (488-oregon green Thermo Fisher Scientific) was conducted after blocking (1% Bovine serum albumin (BSA) + 4% goat serum in TBS-T) and was followed by staining with the appropriate secondary antibody (Alexa Fluor goat anti mouse 488 and goat anti rabbit 594 (Invitrogen #1844440 and #1858182)). For NFATc1(7A6) antibody (Thermo Fisher Scientific #MA3-024), sections were pretreated with the M.O.M. (mouse on mouse) immunodetection kit (Vector Laboratories, #BMK-2202) with deployment of 0.1% Sudan Black/70% ethanol for autofluorescence quenching at the conclusion of the protocol. Cover slips were mounted on glass slides using ProLong Diamond Antifade Mountant with DAPI (Life Technologies) and images were acquired on a Leica SP8 microscope.

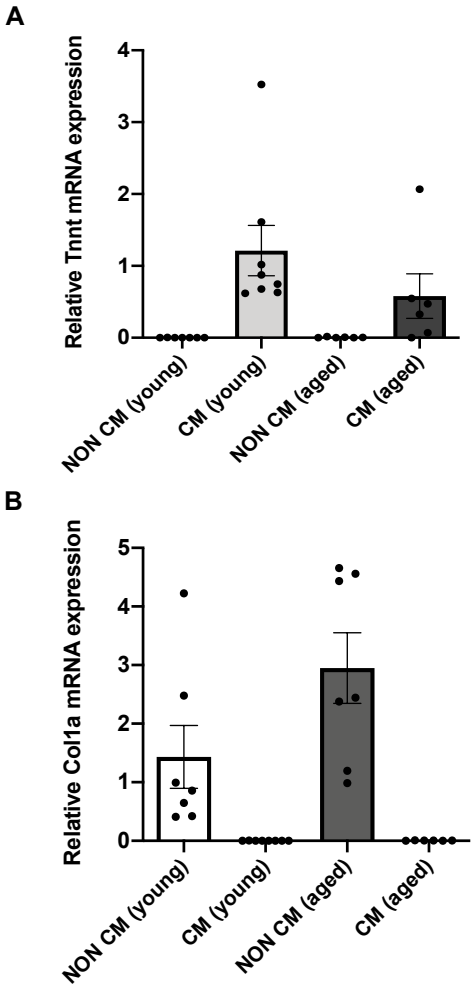
Adult mouse cardiomyocyte and noncardiomyocyte isolation

Cardiomyocytes and noncardiomyocytes were isolated from two- and twenty-months old adult male mice using a Langendorff-free method as previously described [50]. We modified the original protocol to include blebbistatin (5 μM; Sigma Aldrich, catalog #B0560-5MG) instead of 2–3-butanedione monoxime in the culture media, to enhance cell survival. Briefly, mice were anesthetized with isoflurane, and then the chest cavity was opened. The descending aorta and inferior vena cava were cut followed by perfusion of 7 ml of EDTA buffer in the apex of the right ventricle. The aorta was then clamped and cut distal to the clamp, and the heart was removed. EDTA buffer (10 ml) was then perfused in the apex of the left ventricle followed by injection of 3 ml of perfusion buffer and then 30–40 ml of collagenase buffer delivered in the left ventricular apex. The clamp was removed, and the heart was manually dissociated. Stop buffer (perfusion buffer + 5% fetal bovine serum) was added, cells were passed through a 200-μm strainer, and then cardiomyocytes were allowed to gravity settle for 20 min. The supernatant containing noncardiomyocytes and debris was plated in an uncoated tissue culture plate in DMEM/F-12/10%FBS for 3 h (ThermoFisher Scientific, catalog #11320082). The cardiomyocyte fraction in the pellet then underwent sequential gravity settling with low-speed centrifugation (12 *g*, 3 min) with calcium reintroduction followed by plating in laminin-coated plates for 3 h. After 3 h, both cardiomyocytes and noncardiomyocytes were washed and harvested in TRIzol (Qiagen, catalog # 79306). Samples were frozen at −70°C until RNA extraction with phase separation, followed by column purification according to the manufacturer's instructions (Zymo Research).

For culture and adenoviral infection, cells were plated on laminin coated 6-well-dishes in culture media following previously published protocols.[50] We have added AdV-LacZ, AdV-

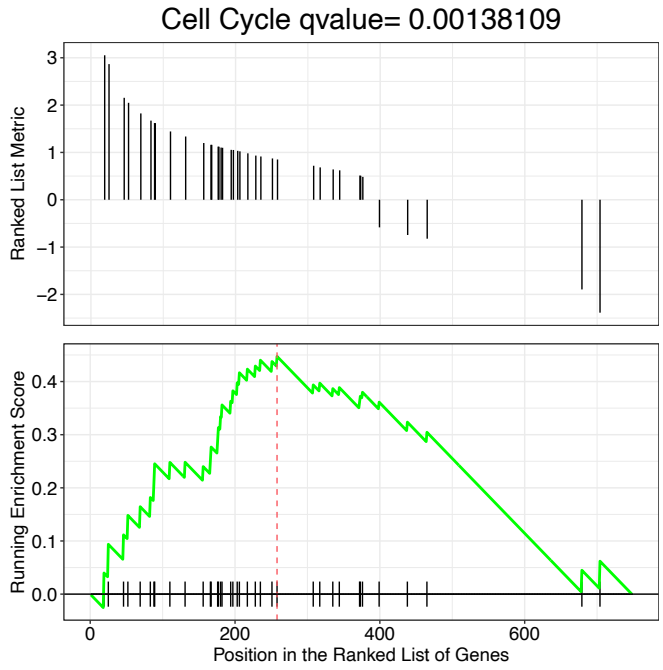
RCAN1.1, and AdV-RCAN1.4 at an MOI of 50 to the culture medium (M199) containing 0,1% BSA, ITS, 10mmol/l BDM, CD lipid and PenStrep for overnight incubation.[50] Cells were carefully washed and subsequently lysed in 600µl Trizol per well and RNA extraction followed by cDNA synthesis and qPCR analyses have been carried out as above.

Supplemental Figures

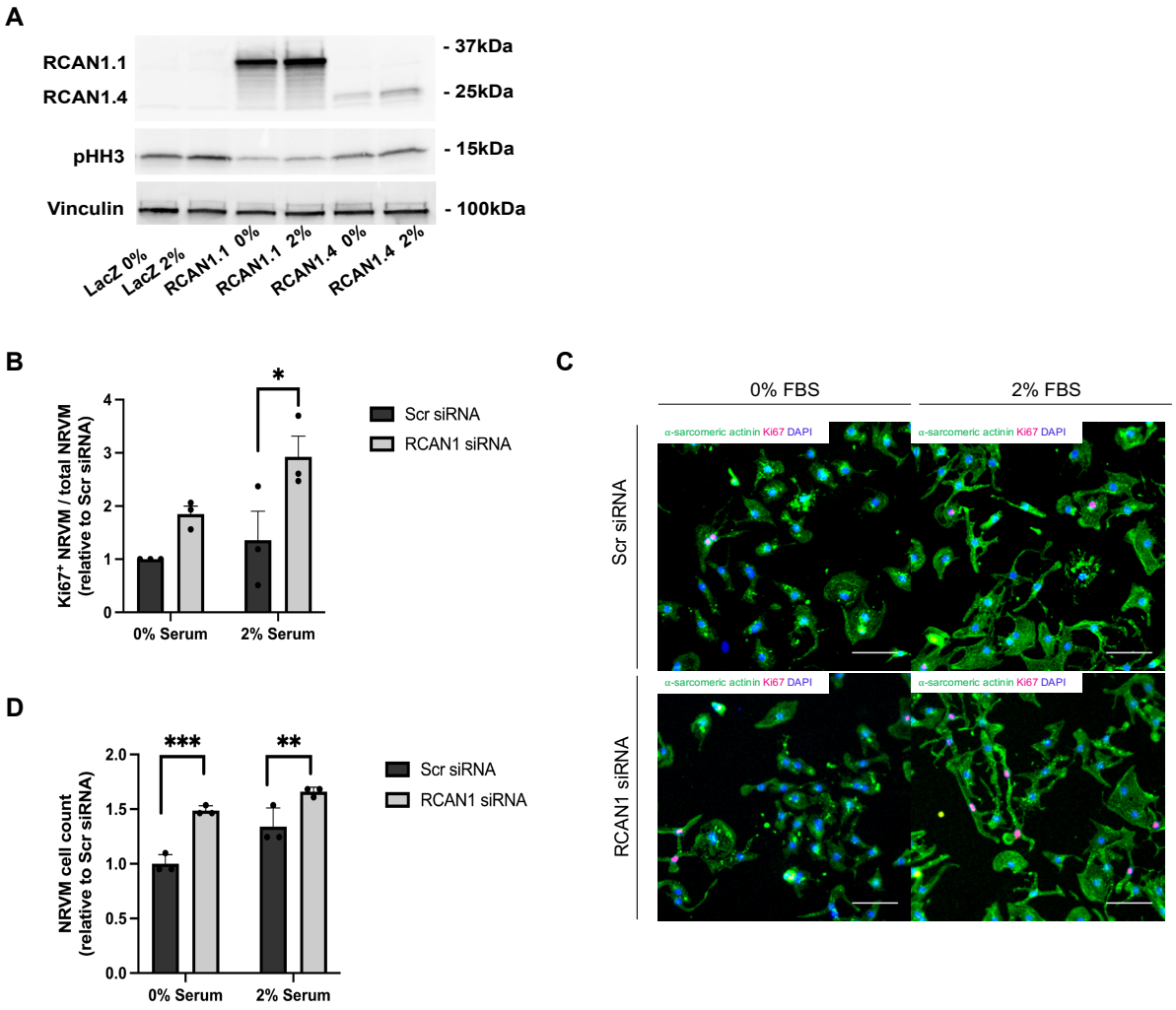


Supplemental Figure S1: Confirmation of cell purity from isolated cell experiments and expression of gene targets in sedentary control mice. The purity of isolated adult cardiomyocyte and noncardiomyocyte fractions from young and aged mice was confirmed by qPCR. **(A)** *Tnnt* expression was detected in cardiomyocytes while **(B)** *Col1a* was expressed in the noncardiomyocyte fraction (n=6-8 mice per group). Data are shown as Mean± s.e.m.

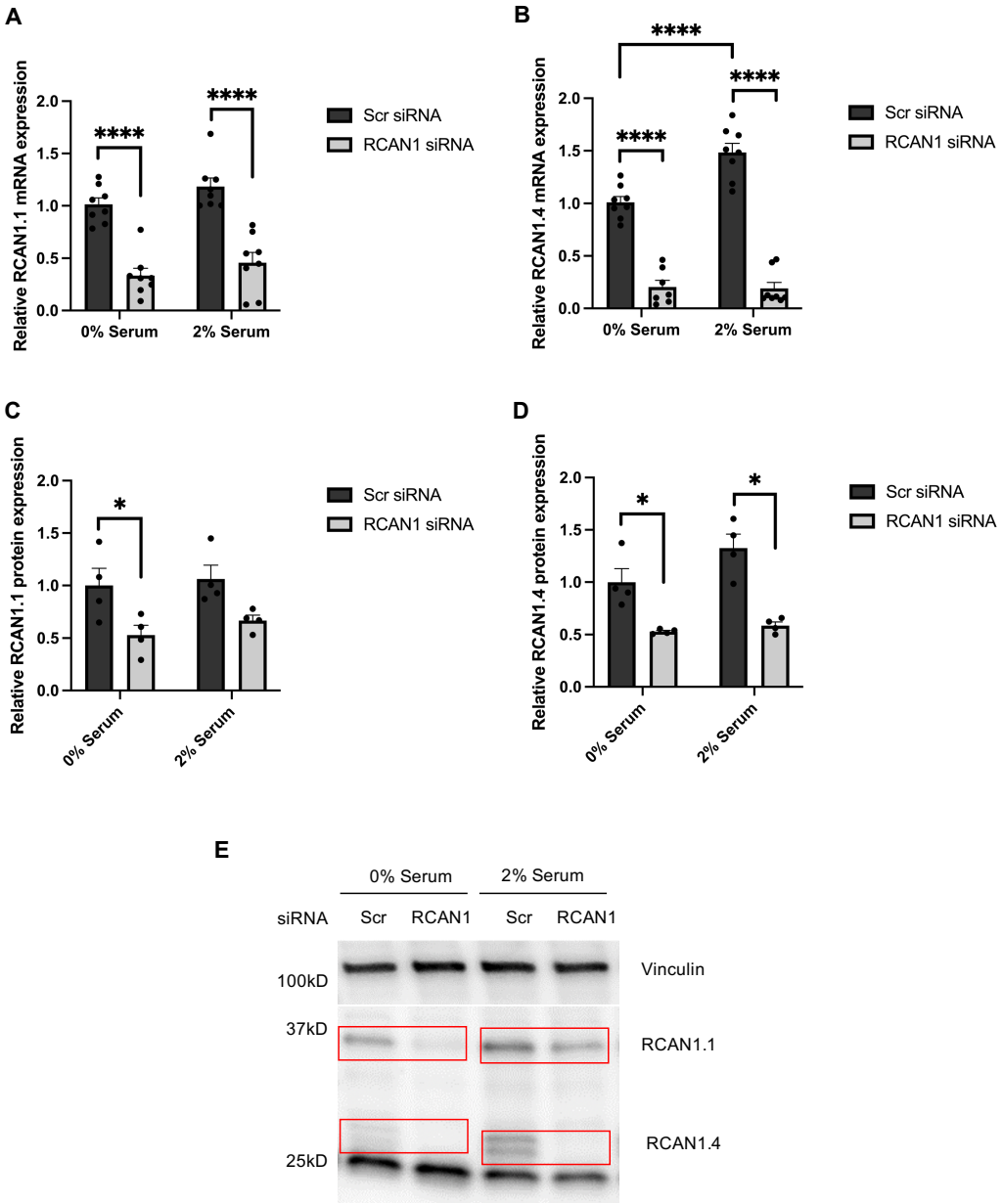
A



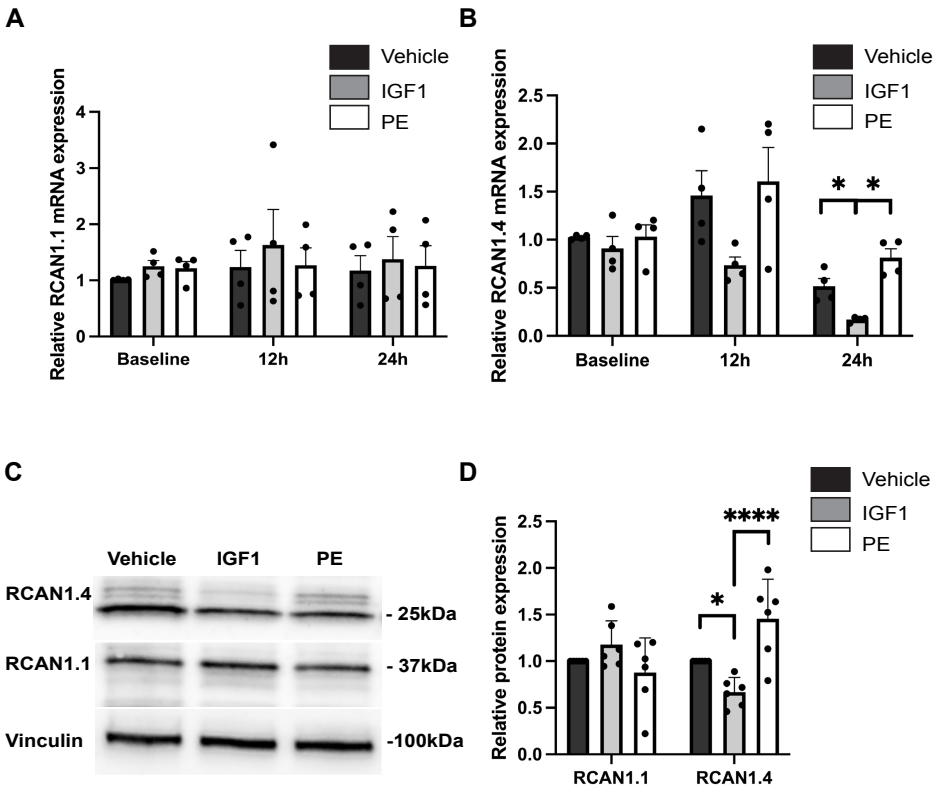
Supplemental Figure S2: Enrichment of Cell cycle genes in aged, exercised hearts. **(A)** Gene set enrichment analysis (GSEA) from our RNAseq dataset shows that Cell cycle genes are significantly upregulated in aged, exercised hearts (interaction age:exercise).



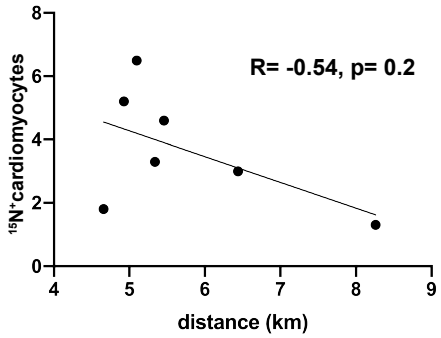
Supplemental Figure S3: Loss of RCAN1 induces proliferation in neonatal cardiomyocytes. (A) Representative immunoblot demonstrating successful overexpression of RCAN1.1 and RCAN1.4 (LacZ was used as control adenovirus) in either serum-free conditions (0%) or in the presence of 2 % serum, as well as respective pHH3 expression in these samples. Vinculin served as protein loading control. **(B-C)** NRVM were transfected with scrambled siRNA (Scr siRNA; served as control) or RCAN1 siRNA and stained for Ki67. Scale bar 50μm. Loss of *Rcan1* resulted in a significant increase in Ki67+ cardiomyocytes (2% serum) and **(D)** an increase in cardiomyocyte cell count (n=3 biological replicates, *p<.05, **p<0.01, ***p<0.001, two-way analysis of variance (ANOVA)). Data are shown as Mean± s.e.m.



Supplemental Figure S4: Deletion of RCAN1 in neonatal cardiomyocytes. RCAN1 siRNA treatment of NRVM, under both serum-free and low serum conditions, resulted in a significant downregulation of both *Rcan1.1* and *Rcan1.4* mRNA (n=7-8 independent replicates/group) **(A** and **B)** and protein levels (n=3 independent replicates/group) **(C** and **D)**. **(E)** Representative immunoblot of RCAN1.1 (37kD) and RCAN1.4 (25kD) protein expression after RCAN1 or Scr control siRNA treatment. Vinculin served as a protein loading control. (* $p<0.05$, **** $p<0.0001$ vs Ctrl by two-way analysis of variance (ANOVA). Data are shown as Mean± s.e.m.



Supplemental Figure S5: RCAN1 expression in response to different stimuli. NRVM were treated with IGF1 or PE as indicated for 12 and 24h and *Rcan1.1* and *Rcan1.4* mRNA was investigated **(A** and **B). (C)** Representative Western Blot of RCAN1.1. and RCAN1.4 protein expression after stimulation with IGF1 and PE as indicated (24h). (**D)** Quantification of RCAN1.1. and RCAN1.4 protein levels after 24h of respective stimulation with PE or IGF1 (n=4-6 independent replicates/group, *p<0.05, ****p<0.0001, two-way analysis of variance (ANOVA)). Data are shown as Mean± s.e.m.



Supplemental Figure S6: Correlation between distance run and cardiomyogenesis in historical cohort of young, exercised mouse hearts. Graph showing no correlation between the number of ^{15}N-thymidine labeled cardiomyocytes and running distance (km) in our cohorts. Each plot represents one mouse (n=7 mice).